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RESEARCH PAPER

Facilely prepared low-density DNA monolayer–based electrochemical biosensor with high detection performance in human serum

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Abstract

Presently, most reported electrochemical biosensors, for highly sensitive and selective detection of nucleic acid, still require multiple, time-consuming assembly steps and high-consumption DNA probes as well as lack good performance in human serum, which greatly limit their applicability. Herein, an easy-to-fabricate electrochemical DNA biosensor constructed by assembly of bovine serum albumin (BSA) followed with direct incubation of amplified products has been proposed. This method combined terminal deoxynucleoside transferase (TdTase)–mediated isothermal amplification and polyHRP catalysis to achieve dual-signal enhancement, and was featured with low-density DNA monolayer for its employment of only 2 nM capture probes. Surprisingly, based on the low-density DNA monolayer, the steric hindrance effect of polyHRP could effectively restrain the background compared with HRP, which further pushes the signal-to-noise (S/N) ratio to 70 than that of most currently available methods. Additionally, this strategy also showed favorable specificity and powerful anti-interference in human serum, and thus potentially attractive for diagnosis of diseases.

Keywords Electrochemical DNA biosensor · Isothermal amplification · Steric hindrance effect · Low-density DNA monolayer

Introduction

Accurate and rapid detection of nucleic acid (DNA/RNA) markers has always been a hot subject for fields like genetic analysis, clinical diagnosis, environmental testing, forensic identification, and anti-bioterrorism [1]. Especially, it is extremely demanding to develop routine diagnosis methods meeting the needs of future point-of-care testing (POCT) [2] and employment in resource-limited settings [3]. Among different types of readout methods (e.g., colorimetry [4, 5], fluorometry [6, 7], electrochemistry [8, 9]), the electrochemical biosensors have attracted considerable interest because of the advantages of simple operation, rapid detection, low cost, and being easy for microminiaturization.

Recently, nucleic acid-based isothermal amplification technologies mediated by enzymes with high catalytic ability, such as rolling circle amplification (RCA) [10, 11], loop-mediated isothermal amplification (LAMP) [12, 13], recombinase polymerase amplification (RPA) [14, 15], and exponential amplification reaction (EXPAR) [16, 17], have been extensively combined to construct highly sensitive electrochemical DNA biosensors for detection of trace-level nucleic acid markers by producing large numbers of DNA copies. Notably, terminal deoxynucleoside transferase (TdTase)–mediated amplification [18] has unique characteristics featured with free of template, multiple enzymes, and complex probe design, which can directly extend DNA fragments along a single-stranded DNA (ssDNA) with 3′-OH terminal under isothermal

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condition. For example, Yang et al. [19] reported an in situ grown DNA nanotail (IGT)–mediated straightforward and template-free signal amplification strategy for highly sensitive and sequence-specific DNA detection with an impressive detection limit of 20 fM. Also, our group [20] proposed a novel TdTase-based electrochemical DNA biosensor employing the grafting to mode, which had significantly improved the detection performance over the in situ polymerization approach–based electrochemical DNA biosensor. Therefore, TdTase had good amplifying performance and promising application in the design of electrochemical DNA biosensor.

Traditionally, the preparation of an electrochemical DNA biosensor involves self-assembly monolayer (SAM) of thiolated ssDNA probe on the Au surface via thiol-gold chemistry overnight, subsequent backfilling with an alkanethiol (e.g., mercaptohexanol, MCH) to reduce non-specific adsorption, and further hybridization with target sequence. However, this protocol often takes a long time (one more day) to prepare functional electrodes and to complete the test. Besides, the multistep analysis process would inevitably increase the random errors. Consequently, a great need exists for rapid, sensitive, and specific strategies avoiding the complicated steps.

In this work, a short-period electrochemical DNA biosensor for sensitive DNA detection has been provided, which was facilely prepared by one-step blocking with bovine serum albumin (BSA) (15 min) and subsequently direct assembly of partial dsDNA products (1 h) generated in homogeneous solution (20 min) by TdTase-mediated extention. As a result, the introduction of multiple biotin-dUTPs along the target DNA and the following specific binding of streptavidin-polyHRP (SA-polyHRP) achieved dual-signal amplification. It is worth noting only 2-nM capture probes that are 3 orders of magnitude lower than those of previously reported studies (μM level of capture probe) were employed in this study, thereby greatly minimizing the working concentration of probes and resulting in a very low-density DNA monolayer. Surprisingly, the results demonstrated that SA-polyHRP, as a specific binding amplifier, had very low background (~ 60 nA) compared with SA-HRP (~ 540 nA) in this low-density DNA monolayer, and signal-to-noise (S/N) ratio as high as 70 could be achieved. This sensor also demonstrated successful trial of TdTase-meditated reaction and product assembly in the presence of human serum, indicating great promise for clinical diagnosis applications.

Experimental sections

Materials and reagents

The BCR/ABL fusion gene, a recognized specific marker of chronic myeloid leukemia, is selected as a proof-of-concept target in this study. All of the DNA nucleotide sequences were purchased from TaKaRa Inc. (http://www.takarabiomed.com.cn/) with their sequences listed as follows:

Capture probe: 3′-SH-$(CH_2)_6$-GTCTCAAGTTTTCGGGAAGTCG-5′
Target: 3′-AGGCGACTTCCCGAAAACTTGAGAC-5′
Single-base mismatched target: 3′-AGGCGACTTCCCGACAACTTGAGAC-5′
Three-base mismatched target: 3′-AGGCGACTCCCCGACAACTTCAGAC-5′
Non-complementary target: 3′-GCTATACCCACTCCGTCATCAGCCT-5′

The synthetic oligonucleotides were dissolved in sterilizing water and stored at − 20 °C.

Bovine serum albumin (BSA) was purchased from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (https://www.sangon.com/). Terminal deoxynucleoside transferase (TdTase), 10× reaction buffer (500 mM CH_3COOK, 200 mM $Mg(CH_3COO)_2$, 100 mM $(HOCH_2)_3CNH_3$-CH_3COOH, pH 7.9), and 10× (2.5 mM) solution of $CoCl_2$ were obtained from New England Biolabs, Ltd. (http://www.neb-china.com/). Streptavidin-HRP conjugate, streptavidin-polyHRP (SA-polyHRP) conjugate, biotinylated 2′-dexyuridine 5′-triphosphate (biotin-dUTP), and 2′-dexyadenosine 5′-triphosphate (dATP) were from Thermo Scientific (http://corporate.thermofisher.com/en/home.html). The TMB (3,3′,5,5′-tetramethylbenzidine) substrate (enhanced K-blue substrate, H_2O_2 included) was purchased from Neogen (http://www.neogen.com/en/).

The buffer solutions involved in this study were as follows: the washing buffer was 10 mM phosphate buffer (PB) (a mixture of 10 mM Na_2HPO_4 and NaH_2PO_4, pH 7.4) and SA-HRP and SA-polyHRP were diluted with 10 mM phosphate buffer saline (PBS) (a mixture of 10 mM Na_2HPO_4 and NaH_2PO_4 including 0.1 M NaCl, pH 7.4) containing 0.25% BSA. The chemicals were of analytical grade and used as received without further purification. All aqueous solutions were prepared and diluted using ultrapure water (18.2 MΩ/cm resistivity) obtained from Millipore Milli-Q system (http://www.merckmillipore.com/).

Preparation of the sensor

In this work, the BSA-based probe carrier platform was fabricated according to the process of literature [21]. Briefly, the gold electrode (2 mm in diameter) underwent physical polishing and electrochemical treatment, and then the cleaned electrode was immersed in 10 mM PB (pH 7.4) containing 0.1 mg mL^{-1} BSA for 15 min at room temperature (25 °C). Meanwhile, a TdTase-mediated extension reaction was carried out in 50-μL volume of solution (5.0-μL reaction buffer, 5.0 μL $CoCl_2$, 0.25 μL biotin-dUTP, 0.75 μL dATP, 0.1 μL

TdTase, 0/5.0 μL target DNA, 5.0-μL capture probe, and 32.9/27.9 μL ultrapure water) for 20 min at 37 °C. After that, the BSA-modified gold electrode was immersed in the above solution for 1 h at 55 °C. After being washed and dried lightly with N_2, the electrode was incubated with SA-polyHRP (1:1000) for 15 min at room temperature. Finally, it was extensively rinsed and subjected to electrochemical measurements.

Electrochemical measurements

A CHI 660E electrochemical workstation (http://www.chinstr.com/) was applied for the cyclic voltammetry (CV) and amperometric experiments. A conventional three-electrode configuration including the modified AuE as the working electrode, a platinum wire as the auxiliary electrode, and an Ag/AgCl as the reference electrode was used throughout the experiments. CV was carried out at a scan rate of 0.1 V s^{-1}. Amperometric detection was performed at a fixed potential of 0.1 V and a steady state was reached and recorded within 100 s in the TMB substrate.

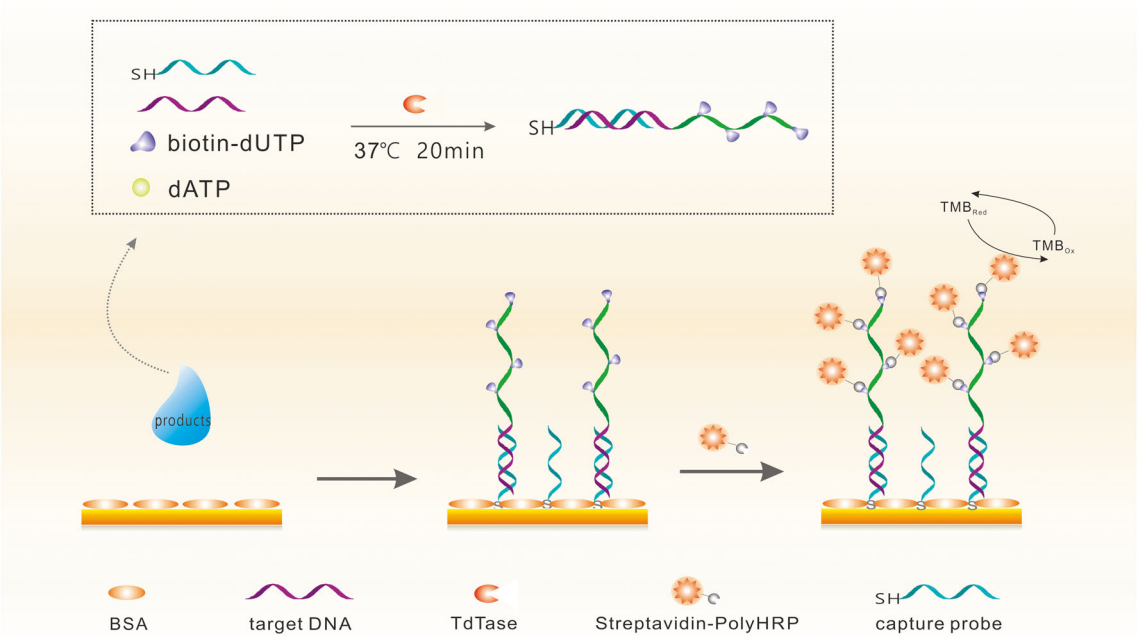
Results and discussion

Working principle of the low-density DNA monolayer–based electrochemical biosensor

The proposed strategy was schematically depicted in Scheme 1. First of all, BSA molecules were self-assembled on the Au electrode to form a porous monolayer. At the same time, the TdTase-mediated extension reaction was performed in homogeneous solution, in which the capture probe labeled with thiols at its 3′ terminals was hybridized with target DNA, forming dsDNA with 3′-OH exposed to TdTase, while no exposure of 3′-OH in the absence of the target DNA. After TdTase polymerization, a dsDNA product with an overhang incorporated with biotin labels was obtained. Due to the dsDNA rigid structure and its easier access to inter-protein pores, the generated product could be rapidly inserted into gaps between BSA via Au–S bond at the dsDNA terminal, resulting in uniform distribution of DNA on BSA monolayer with the optimal spacing, so that reproducibility could be greatly improved. Then, SA-polyHRP could specifically bond to the long generated ssDNA via a biotin-avidin bridge. Finally, we investigated the electric current generated by HRP-catalyzed reduction of H_2O_2 contained in the TMB substrate for quantitative readout of the target DNA.

Characterization for detection of target DNA

Electrochemical impedance spectroscopy (EIS) was firstly carried out to characterize the successive modifications on the gold electrode surface. As shown in Fig. S1 in the Electronic Supplementary Material (ESM), the increasing value of R_{et} further demonstrated the successful modification of BSA, TdTase-mediated product, and SA-polyHRP on the gold electrode surface. Furthermore, the capture probe was also proven to be successfully immobilized onto the BSA-modified gold electrode through Au–S bond (ESM Fig. S2).



Scheme 1 The low-density DNA monolayer–based electrochemical biosensor

Besides, the protein adsorption process was monitored by CV (ESM Fig. S3), and the investigation of the electron transfer reactivity of TMB at the BSA-conjugated surface fully verified the formation of porous monolayer of BSA on gold electrode (Fig. S4). The enzyme-based electrocatalytic process was also characterized by CV and amperometry. As shown in Fig. 1a, two pairs of characteristic peaks were observed that were ascribed to the typical two-electron redox reaction of TMB. In the presence of target DNA (1 nM), there was an apparent increase at the HRP-catalyzed reduction peak (~ 0.25 V) which indicated the successful attachment of SA-polyHRP to the electrode surface. To quantitatively measure the target binding event, amperometry was performed (Fig. 1b), and it was observed that a significant increase in the amperometric current (~4000 nA) in the presence of 1 nM target DNA, while only 60 nA, was obtained for blank (a buffer solution with no target), which demonstrated the validity of this electrochemical DNA sensor.

Optimization of the experimental conditions

In order to obtain the optimal performance of the electrochemical sensor, we further investigated the experimental conditions. Certainly, the concentration of capture probe would be a key factor that will affect the formation and assembly of DNA products on the BSA-based sensing platform. As shown in Fig. 2a, the current derived from 1 nM target DNA appeared parabola-like in the range from 0.2 to 10 nM: low concentration of capture probe hybridized less target DNA resulting in low signal gain and higher concentration of capture probe could capture more target DNAs bringing enhanced electrochemical signal, but an excess of unhybridized capture probe would inevitably compete with dsDNA on the surface of gold electrode, resulting in lower current. It must be pointed out that signal from target was still significantly differentiated from that of blank, even if the concentration of capture probe was 5 times that of target DNA, demonstrating the assumption of a stronger assembly ability of dsDNA than ssDNA for its rigid structure and easier accessibility to inter-protein pores. As a result, the optimized concentration of capture probe was found to be 2 nM. Also, the assembly temperature was finely investigated to ensure that dsDNA would not be denatured into ssDNA but with fast assembly. Results showed the maximum current was observed at 55 °C (Fig. 2b). Besides, some key components including ratio of biotin-dUTP/dATP, amount of employed TdTase, and incubation time in the TdTase-mediated extension reaction were elaborately adjusted, and their optimum conditions were 1/3, 2 U, and 20 min, respectively (Fig. 2c–e).

Superiority of polyHRP over HRP in low-density DNA monolayer

Recently, a chemically engineered polyHRP conjugate was developed to replace the traditional monomeric HRP as a novel amplifier for highly sensitive detection of antibodies and nucleic acids with increased sensitivity. Herein, polyHRP, a supermolecular polymer of HRP containing up to 400 enzyme molecules at maximum, conjugated with streptavidin, was adopted as a tracer. Apparently, the S/N ratio results (Fig. 3a) in a series of different concentrations of target DNA demonstrated an overwhelming advantage of polyHRP over HRP, resulting in sensitivity improvements of roughly 2 orders of magnitude. In addition, it was surprising to find that the background with polyHRP as a tracer was

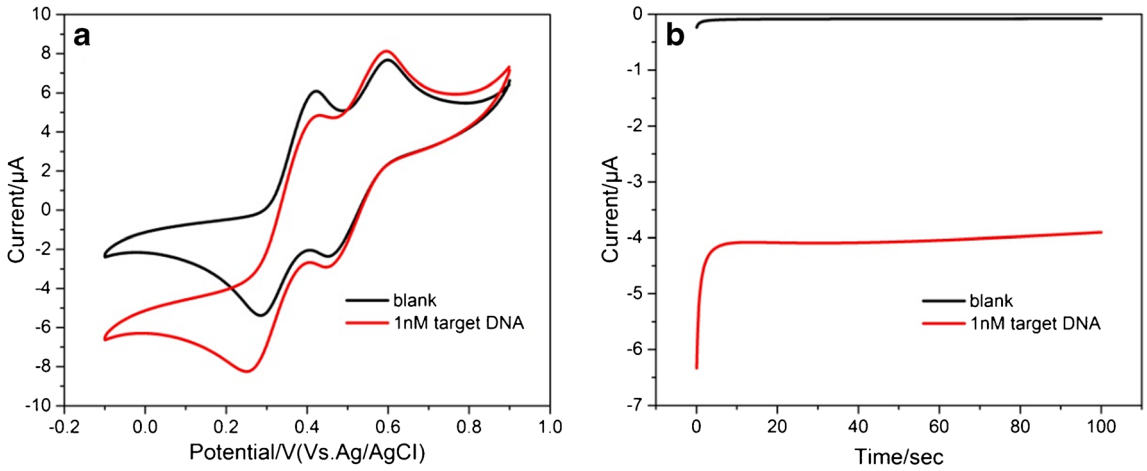


Fig. 1 Cyclic voltammograms (CVs) (**a**) and amperometry (**b**) for the electrochemical DNA sensor based on TdTase-mediated extention and polyHRP binding in the absence (black curve) and presence (red curve) of 1 nM target DNA. The CV curves were measured between − 0.2 and 0.9 V with a scan rate of 0.1 V s^{-1}. The potential of amperometry was held at 0.1 V (vs Ag/AgCl) and the reduction current was recorded at 100 s

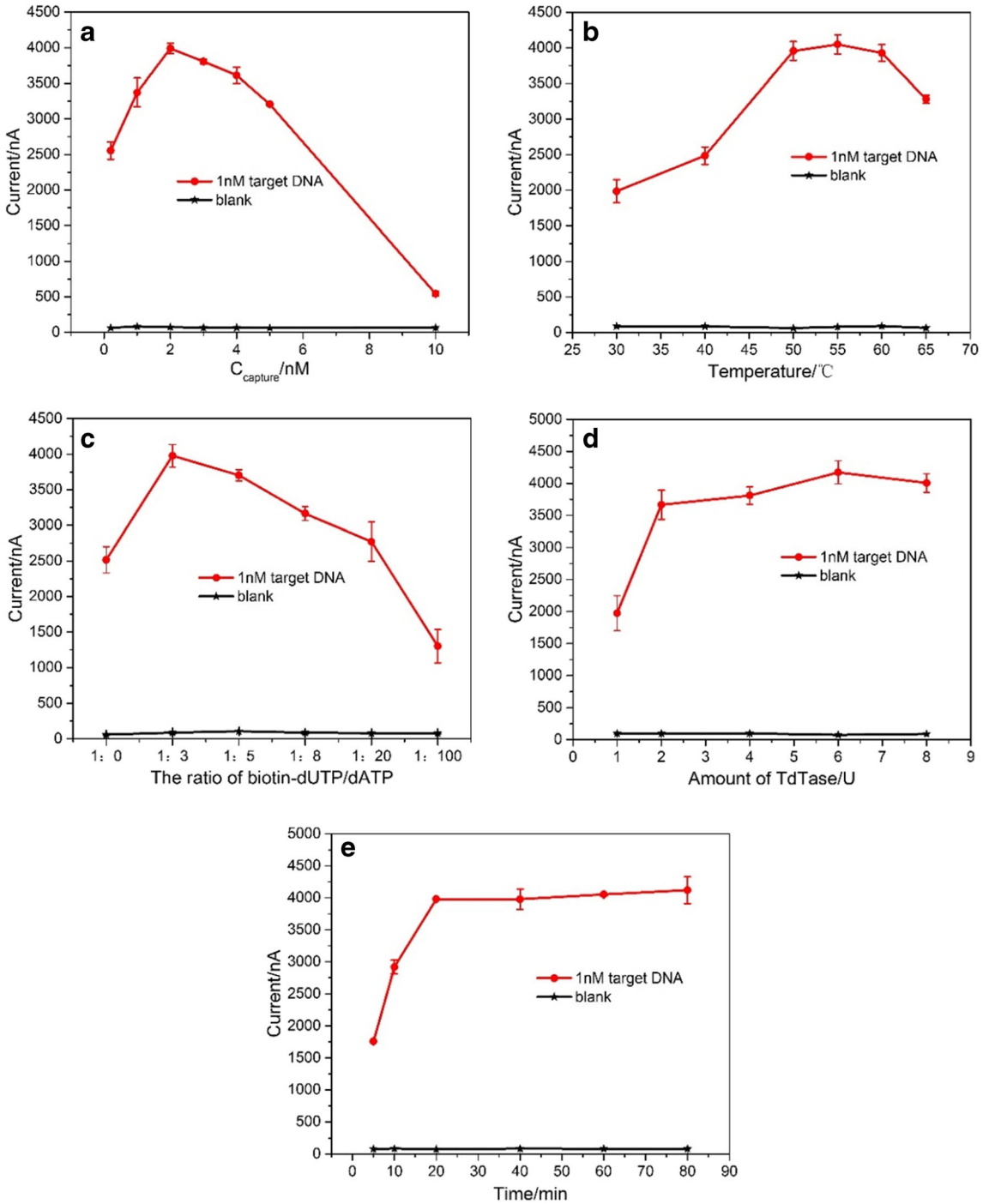


Fig. 2 Effects of (**a**) the concentration of capture probe, (**b**) the assembly temperature, (**c**) the ratio of biotin-dUTP/dATP, (**d**) the amount of employed TdTase, and (**e**) the incubation time on the amperometric response in the presence of 1 nM target DNA (red line) and in the absence of target DNA (black line). Error bars represent standard deviations of measurements ($n = 3$)

significantly lower than that of HRP. For this phenomenon, we tentatively believe it was due to steric hindrance effect caused by the bigger size of polyHRP, leading to its difficulty in passing through DNA monolayer to reach BSA monolayer, producing non-specific adsorption. In order to verify our speculation, experiments about the effect of density of DNA monolayer on background were performed, where different concentrations of capture probe (1 nM, 2 nM, 10 nM, 20 nM, 60 nM, and 100 nM) were used to control the density of DNA monolayer. As shown in Fig. 3b, the background current decreased obviously until a plateau with increasing concentration of capture probe or when HRP as a tracer while

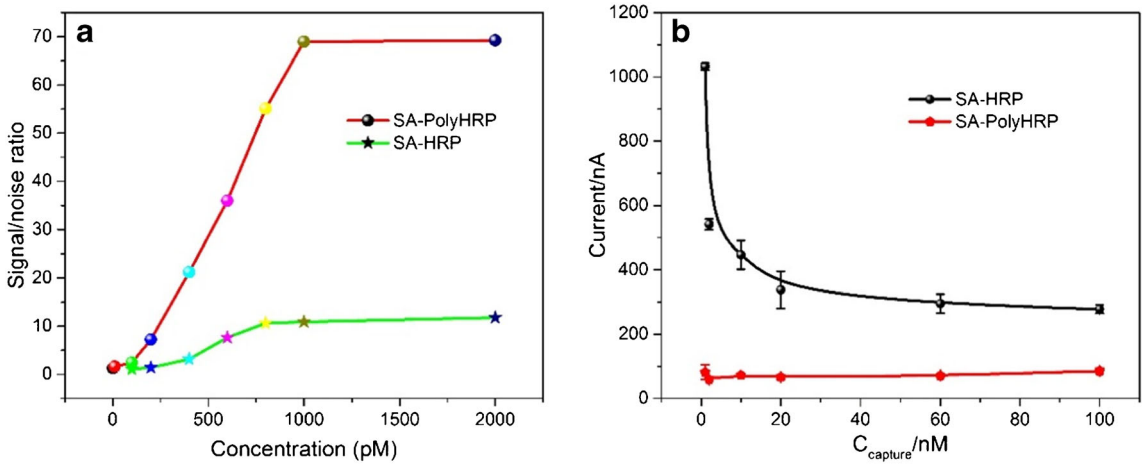


Fig. 3 (**a**) Comparison of S/N ratio between SA-polyHRP and SA-HRP in the sensor, where the S/N ratio is short for signal-to-noise ratio, which was calculated as follows: $S/N = I_{signal}/I_{blank}$. (**b**) Comparison of curves (amperometric signal vs concentration of the capture probe in the absence of target DNA) between SA-HRP and SA-polyHRP. Error bars represent standard deviations of measurements ($n = 3$)

a horizontal line was observed in polyHRP situation, confirming the steric hindrance effect, especially when the concentration of the capture probe was 1 nM; the current responses from HRP and polyHRP were 1000 nA and 60 nA, respectively. Therefore, polyHRP as a tracer exhibited extremely improved sensitivity than HRP not only through enhancing the loading of enzyme labels but also by suppressing the background, particularly in the low-density DNA monolayer interface. Comparisons of the S/N ratio between this sensor and other reported electrochemical strategies using amperometric detection in the HRP-TMB-H_2O_2 system are displayed in Table 1, showing significant advantage of our proposed strategy over most currently available methods, especially in terms of simplicity of operation, time, and probe cost.

Analytical performance of the sensor

Different concentrations of target DNA were detected under the optional experimental conditions. As shown in Fig. 4a, the signal response increased with the increasing concentration of target, and there was a good linear relationship between current and target DNA concentration in the range of 100~1000 pM with a correlation equation of $y = 4.476x - 436.666$ ($R = 0.998$) (Fig. 4b). The LOD was estimated to be 1 pM (> 3SD) (ESM Fig. S5). Also, this electrochemistry assay showed a good reproducibility, which was manifested by a relative standard deviation (RSD) of 5% for three repetitive measurements of 1.0 nM target with separate electrodes.

In addition to sensitivity, selectivity was another significant performance indicator for sensors. Then, we

Table 1 Comparison of different electrochemical DNA biosensors using amperometric detection in the HRP-TMB-H_2O_2 system

Strategy	Background current (nA)	S/N	Probe concentration (μM)	Experimental period (h)	Reference
SIEP	~ 700	4.5 (1 μM target)	1	> 5 h	[22]
HCR combined with DNA tetrahedral nanostructure	5	150 (1 nM target)	1	> 12 h	[23]
NIEP	187	~ 6.5 (1 nM target)	0.5	> 12 h	[24]
dSH-HSP	~ 100	60.7 (5 nM target)	1	~ 2.5 h	[25]
Allosteric DNA tetrahedral bioprobes	120	5 (0.1 nM target)	1	> 12 h	[26]
Poly-adenine-mediated DNA self-assembly	32	42.5 (1 nM target)	0.1	> 12 h	[27]
DTT-based surface monolayers	6	327.2 (1 nM target)	0.05	> 12 h	[28]
HDT-based surface monolayers	10	344.9 (1 nM target)	0.05	> 12 h	[29]
TdTase-mediated extention and polyHRP binding	60	70 (1 nM target)	0.002	< 2 h	This study

SIEP, HCR, NIEP, dSH-HSP, DTT, and HDT are abbreviations of surface-initiated enzymatic polymerization, hybridization chain reaction, nanoprobe-initiated enzymatic polymerization, dual-thiolated hairpin structured DNA probe, dithiothreitol, and hexanedithiol respectively

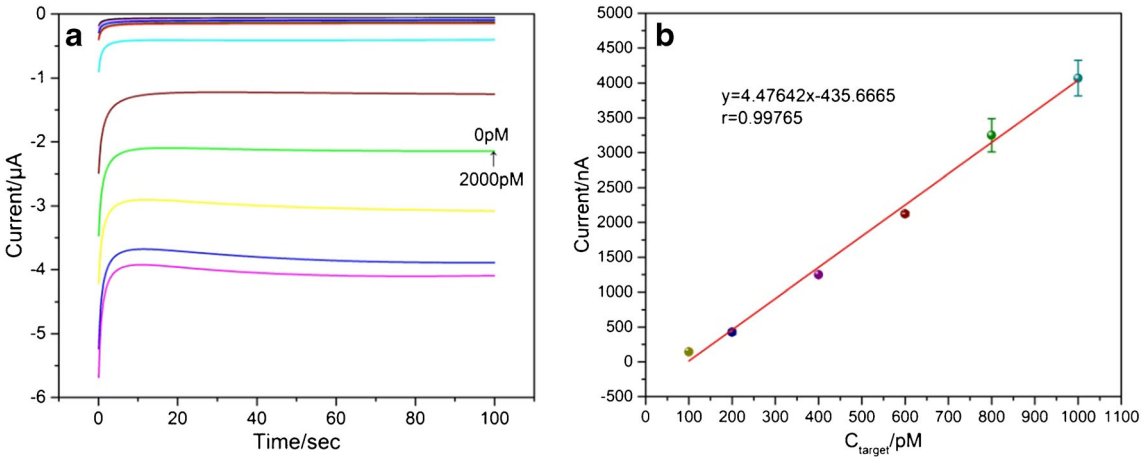


Fig. 4 (**a**) Amperometric curves of target DNA with a series of concentrations (0, 1 pM, 5 pM, 10 pM, 40 pM, 100 pM, 200 pM, 400 pM, 600 pM, 800 pM, 1000 pM, and 2000 pM) in TMB substrate solution. Potential of amperometry was 0.1 V. (**b**) A calibration plot of the amperometric current vs concentration of target. Data were collected from at least three independent sets of experiments

evaluated the discrimination capability of the TdTase-mediated and polyHRP binding–based electrochemical DNA biosensor by using different oligonucleotides, including single-base mismatched (SM) target, three-base mismatched (TM) target, and non-complementary (NC) target. It was found that all the mismatched sequences could be readily distinguished from the fully complementary DNA (Fig. 5), and even for the single-base mismatched DNA, the current was clearly distinguished from the complementary target. Hence, these results indicated that this method displayed good selectivity for the determination of target DNA in the presence of certain amount of interference. However, the obtained response will not discriminate the signals between complementary DNA and mismatched targets if the mixture of DNA samples would contain all of these tested targets with various concentrations, which was a challenging issue that deserved to be studied in our further research. Thus, results shown here together with the aforementioned comparisons in Table 1 significantly demonstrate the proposed low-density DNA monolayer–based electrochemical biosensor is facile, cost-effective, and high-performance.

Detection of target DNA in the presence of human serum

In order to assess the applicability of the new electrochemical genosensor to biological fluids, we further

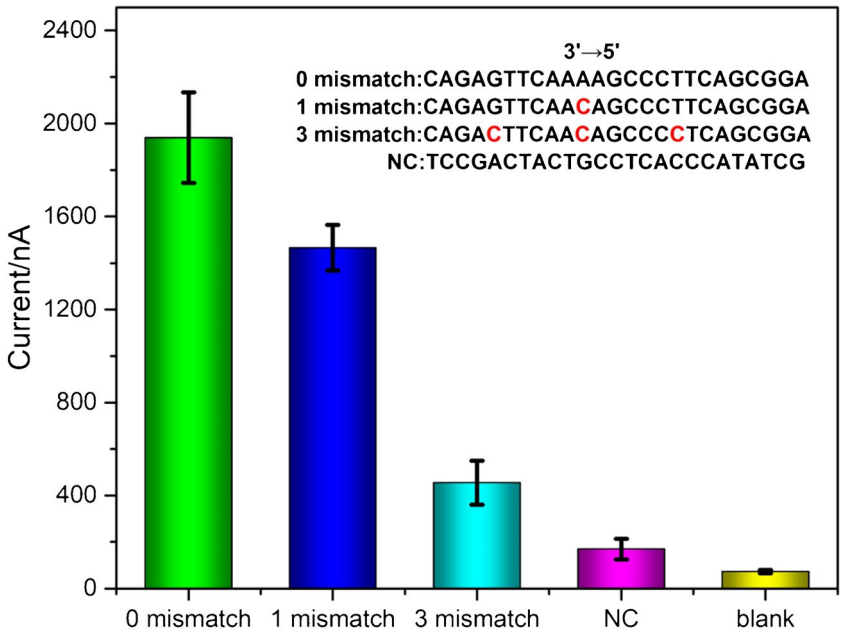


Fig. 5 Selective response of the sensor to different DNA sequences, including complementary target, single-base mismatched target, three-base mismatched target, non-complementary target, and control (blank). All the sequences were in equal concentration at 500 pM. Error bars represent standard deviations of measurements ($n = 3$)

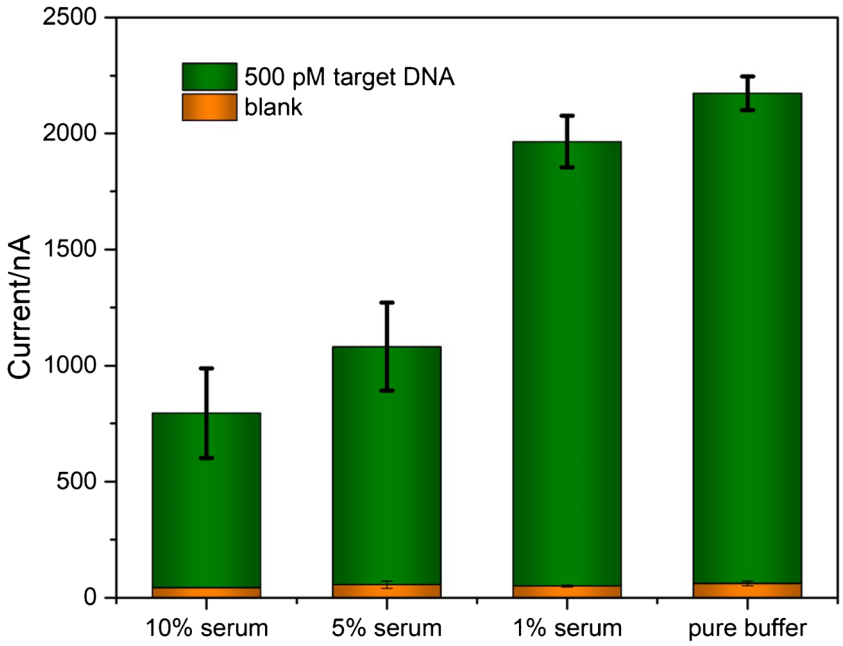


Fig. 6 Amperometric responses for the TdTase-mediated extention and polyHRP binding sensor challenged with target DNA in pure buffer and human serum. Error bars represent standard deviations of measurements ($n = 3$)

conducted its target detection in the presence of human serum. Human serum as a realistically complex matrix is known to inhibit or even terminate mostly enzymatic reactions like qRT-PCR. Herein, the TdTase-mediated extention reaction containing 0%, 1%, 5%, or 10% human serum with or without target was performed, followed by assembly on BSA-modified electrodes and final electrochemical measurements. As shown in Fig. 6, the TdTase-mediated extension could be effectively conducted in a certain diluted serum while most enzymatic reactions like qRT-PCR completely could not perform in it, which suggested that the TdTase-mediated enzymatic isothermal amplification-based electrochemical DNA biosensor had great potential in detecting target of interest in serum samples, and in this regard, this approach will be feasible to detecting relevant circulating DNAs in complex biological fluid previous target DNA extraction. Therefore, these results suggest that the TdTase-mediated and polyHRP binding–based electrochemical DNA biosensor showed great promise as a practical application for direct detection of nucleic acid in real samples.

Conclusions

In summary, a facile electrochemical DNA biosensor was prepared by one-step blocking and one-step assembly method, which was featured by low-density DNA monolayer with introduction of multiple signal molecules. Dual-signal amplification strategy, using fast isothermal TdTase-mediated incorporation in homogeneous solution and the following specific binding of polyHRP, could greatly improve the sensitivity of the biosensor. Significantly, compared with HRP, the steric hindrance effect by polyHRP effectively suppressed the background, particularly in this low-density DNA monolayer, further enhancing the S/N ratio as high as 70. Thus, the proposed strategy was rapid, sensitive, and economical and has low background and high anti-interference ability in human serum. With the above advantages, this method displayed great application potential in the clinical diagnostics and point-of-care testing.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical standards and informed consent This study conformed to the ethical guidelines of the Declaration of Helsinki and was approved by The Ethics Committee for Human Research, The First Affiliated Hospital of Fujian Medical University. Human serum samples used in this study do not have any identifying information about all the participants that provided written informed consent.

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